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Original Article

Optimization for extraction of an oil recipe consisting of white pepper, long pepper, cinnamon, saffron, and myrrh by supercritical carbon dioxide and the protective effects against oxygen–glucose deprivation in PC12 cells

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ABSTRACT

This study is to investigate the most efficient extractives of extracting oil recipe for stroke treatment and the protective effects on an oxygen and glucose deprivation model in PC12 cells. An orthogonal experimental design L_9 (3^4) was carried out for oil recipe's optimization with supercritical CO_2 fluid extraction. 2-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and enzyme-linked immunosorbent assay were conducted to evaluate cell activity and indexes in the cell lysate. The result showed that the optimum extraction condition was 30 Mpa, 50 °C, 100 min, the extracts were analyzed by gas chromatography–mass spectrometry and among forty detected compounds 27 were identified, representing 80.86% of the total oil content. *trans*-Cinnamaldehyde (14.14%), piperine (9.32%), β-amyrin (6.79%), lupenone (6.28%), longifolene (6.07%), β-caryophyllene (5.21%), α-bisabolol (4.11%), and β-bisabolene (2.56%) were high mass fraction. Oil recipe could significantly attenuate PC12 cell damage, the lactate dehydrogenase release and decreased the malondialdehyde levels, glutathione peroxidase and nicotinamide adenine dinucleotide phosphate oxidase activity, glutathione and nitric oxide content ($p < 0.01$) and increased the level of superoxide dismutase after oxygen and glucose deprivation. The protective mechanism may be related to oil recipe's antioxidant effect by scavenging free radicals.

Introduction

The country incorporated national characteristics into the medical system in China, including the traditional Chinese Hui medicine (TCHM) which is a representative folk medicine and an important branch of traditional Chinese medicine. The most characteristic of the TCHM is the aromatic drug mainly due to rich oil, and usually emits aromatic smell. Stroke is the third most frequent cause of adult death in most industrialized countries after cardiovascular disease and cancer and has been a serious threat to human health (Flynn et al., 2008). It is important to develop the effective treatment of neuroprotective drugs, due to few treatments for stroke and the imperative development of new therapeutics. Aromatic drugs in prescriptions for cerebral ischemia have been studied and found with remarkable treatment effects (Qi et al., 2016). *Trans*-cinnamaldehyde has been confirmed that played a major role in ameliorating cerebral ischemia-induced brain injury and protected myocardial ischemia damage (Zhao et al., 2015; Chen et al., 2016).

Our previous research show that treating cerebral stroke contains white pepper, long pepper, cinnamon, saffron, and myrrh as the main active components (equivalent to the monarch in the traditional Chinese medicine prescription) in TCHM (Li et al., 2013a,b). The main active components have many pharmacological effects, such as activating blood and dissolving stasis, anti platelet aggregation and prevention and treatment of atherosclerosis (Bai and Xu, 2000; Feng, 2004). Preliminary results show that oil recipe (OR) exert anti-inflammatory and analgesic effect (Zhang et al., 2014), which is key components of the mechanisms for treating ischemic

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stroke and other forms of ischemic brain injury (Jin et al., 2013; Shichita et al., 2012). However, no direct evidence has confirmed that OR exerts a protective effect on cerebral ischemia. Rat pheochromocytoma PC12 cells, which are often adopted as *in vitro* cell culture model of ischemia, have been widely used as a cellular model for studying neuronal diseases (Ryu et al., 2014). Compared with the traditional extraction method, the application of supercritical CO_2 fluid extraction (SFE) has lots of advantages, such as fast extraction rate, avoiding heat deterioration and no residual solvent (Uquiche et al., 2015). In the present study, we verified the protective effect of OR against oxygen and glucose deprivation (OGD) in PC12 cells and thus provided scientific basis for the further development of drugs for stroke treatment.

Materials and methods

White pepper (*Piper nigrum* L., Piperaceae), long pepper (*Piper longum* L., Piperaceae), myrrh (*Commiphora myrrha* (Ness.) Engl., Burceraceae), cinnamon (*Cinnamomum cassia* (L.) J.Presl, Lauraceae), and saffron (*Crocus sativus* L., Iridaceae) were purchased from Taiyuan Herbal Medicine Co., Ltd. (Anhui, China). All of these plants were classified by Lin Dong at the Pharmacognosy Department, College of Pharmacy, Ningxia Medical University, and voucher specimens were deposited in one unit (Herbarium number: 20130721, 20130723, 20130801, 20130811, 20130813).

The reagents, materials, and apparatuses used were as follows: *trans*-cinnamaldehyde reference substance (Chinese Food and Drug Inspection Institute, Voucher No.: 110710-201418, 99.4%); supercritical CO_2 extraction unit (US Applied Separations Inc.); 6890N Agilent gas chromatograph (American Agilent Company); gas chromatography–mass spectrometry (GC–MS) apparatus (American Agilent Company); anaerobic bag (Mitsubishi Ltd.); PC12 cell line (Shanghai Institute of Chinese Academy of Sciences); carbon dioxide (CO_2, 99.95%); high-glucose Dulbecco's modified Eagle's medium (DMEM; Hyclon); PBS (Hyclon); fetal bovine serum (FBS; PAN); nimodipine (Sigma); glucose-free culture medium (Gibco); commercial kits for lactate dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GPX), nicotinamide adenine dinucleotide phosphate oxidase (NOX), and nitric oxide (NO) (Beijing Chenglin Bioengineering Institute). The remaining materials used were analytical reagents.

Oil extraction

After proper crushing and sieving in 40 mesh, the white pepper, long pepper, myrrh, cinnamon, and saffron were mixed at the conventional ratio 1:2:2:2:2 (w/w). Then, 45 g sample was loaded into the extraction cell. Carbon dioxide played a cooled and liquefied role which from a gas cylinder passed to refrigerator unit. In the high pressure pump the liquefied CO_2 was compressed, then passed into a surge tank and transferred to the main extraction column. The other conditions were maintained to be constant within the different experimental conditions was tested in triplicate. Percentage content of oil can be calculated on basis of the dried powder weight (Sodeifian et al., 2017).

$$\text{OR yield}\,(\%) = \frac{\text{OR weight}\,(\text{g})}{\text{material loaded}\,(\text{g})} \times 100\%$$

Determination of cinnamonaldehyde content

GC was performed using an Agilent 6890N gas chromatograph, HP-5MS quartz capillary column (30.0 m × 320 μm × 0.25 μm), and carrier gas for high-purity nitrogen with 1.2 ml/min column flow. The heating program included a column temperature of 50 °C for 1 min; a temperature ramp of 8 °C/min to 150 °C, which was held for 3 min; a temperature ramp of 3 °C/min to 210 °C, which was held for 5 min; and finally, a temperature ramp of 5 °C/min to 240 °C, which was held for 15 min. The inlet temperature was 250 °C, the temperature of the detector was 280 °C, and the sample volume was 0.2 μl. The split ratio was 20:1.

Standard curve preparation for trans*-cinnamaldehyde*

trans-Cinnamaldehyde reference substance (CRS) was dissolved in methanol to produce a solution containing 2.98 mg/ml of the substance as the reference solution. The control products above were diluted 5, 10, 20, 30, and 40 times, generating a series of standard solutions and triplicates for each sample. These solutions were then delivered separately into the gas chromatograph for analysis. The measured average peak area Y corresponds to the vertical coordinate, and the *trans*-cinnamaldehyde concentration X is in the horizontal coordinate. A standard curve of *trans*-cinnamaldehyde (Deng et al., 2014) content $y = 233.76x - 4.1337$, with $r = 0.9999$, was obtained. The linear relationship between 0.0745 and 2.98 mg/ml was favorable.

Single-factor experiment

To determine a reasonable range for the orthogonal experiment, we performed single-factor tests for the factors affecting the OR yield, namely, extraction pressure, temperature, and time. When a certain factor was investigated, other factors became unchanged.

Orthogonal experiment

Given the single-factor experimental results and the OR yield and *trans*-cinnamaldehyde content as indices, selected extraction temperature A (45 °C, 50 °C, and 55 °C), extraction pressure B (20, 25, and 30 Mpa), and extraction time C (80, 100, and 120 min) as three factors for the factor level. Using an $L_9\ (3^4)$ orthogonal experimental design, investigated the influence of the three factors on the OR extraction yield and the *trans*-cinnamaldehyde content obtained by SFE.

$$trans\text{-Cinnamaldehyde}\,(\text{mg/g}) = \frac{trans\text{-cinnamaldehyde}\,(\text{mg})}{\text{material loaded}\,(\text{g})}$$

In the composite-grade method, the weight assignment for the OR yield was 0.3, whereas that for *trans*-cinnamaldehyde was 0.7. The specific calculation method was applied in nine samples. The extraction yield of each sample or the content of the *trans*-cinnamaldehyde was divided by the extraction yield of nine samples or the maximum value of the content of *trans*-cinnamaldehyde, and then the weight coefficient was multiplied. The contribution value of the composite grade of extraction yield or the content of *trans*-cinnamaldehyde in the sample was obtained. The sum of the extraction yield and contribution value of the *trans*-cinnamaldehyde content in the same sample was regarded as the composite grade of the sample.

OR extraction and analysis

The appropriate amount of oil at the optimal extraction process, obtained the total ion chromatogram through the automatic request of spectral graphs from GC–MS. Comparing the mass spectra with those of authentic compounds previously analyzed and stored in the database from the national institute of standards and technology, the components were identified individual (Sylvestre et al., 2006; Cheng et al., 2008; Argyropoulou and Skaltsa, 2012; Chen et al., 2013; Murugan and Mallavarapu, 2013; Singh